Metabolic Changes in Human Red Cells during Incubation of Whole Blood *in vitro*

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1. Whole blood was incubated at 37°, while being dialysed against a large volume of iso-osmotic bicarbonate buffer, pH 7.4. The buffer contained glucose and the essential inorganic components of blood plasma in proportion. 2. After 3 hr. of incubation *in vitro* there is a loss of red-cell 2,3-diphosphoglycerate. 3. Isotope experiments show that this is due to an accelerated rate of destruction of this compound. 4. Simultaneously, there is an increase in the median of red-cell osmotic fragility. 5. After extended periods of incubation there is a decrease in the metabolic rate and a decrease in the ratio of the rates of lactate production to glucose consumption. 6. There is a continuous loss of total adenine nucleotide and, after the first 12 hr. of incubation, a tendency for the intracellular Na⁺ and K⁺ to equilibrate with the plasma. 7. The standard deviation of red-cell osmotic fragility expressed among the red-cell population increases exponentially with the time of incubation.

Red cells have a life-span *in vivo* of about 120 days. During this time they undergo metabolic changes that are thought to determine the aging process. The most important of these changes is probably the decrease in the activity of glyceraldehyde 3-phosphate dehydrogenase, which is accompanied by a loss of ATP (Löhr, Waller, Karges, Schlegel & Müller, 1958). When blood is withdrawn from the body the cells deteriorate rapidly and eventually haemolyse. A rapid deterioration of the red cells also occurs when they are subjected to static conditions *in vivo*; this occurs when red cells in dialysis sacs are placed intraperitoneally or when blood is injected into the body cavities (Motulsky, Casser, Giblett, Broun & Finch, 1958*a,b*), and also when the red cells are subjected to stasis in the spleen (Emerson, Shen, Ham, Fleming & Castle, 1956) or when there is a considerable degree of intravascular stasis. The similarities between blood subjected to static conditions *in vivo* and incubation *in vitro* have been described (Ham & Castle, 1940). The mechanisms, and the order of events involved in the reported deterioration and consequent haemolysis of red cells during incubation *in vitro*, do not appear to have been described.

In some genetically determined haemolytic diseases, the instability of the red cell is due to defects in metabolism. In some cases these defects have been identified, e.g. red cells deficient in

pyruvate kinase, triose phosphate isomerase or glucose 6-phosphate dehydrogenase have an increased tendency to haemolyse *in vivo* (Tanaka, Valentine & Miwa, 1962; Schneider, Valentine, Hattori & Heins, 1965; Carson, Flanagan, Lakes & Alving, 1956). Similarly it ought to be possible to relate the changes occurring during incubation *in vitro* to specific changes in the biochemistry of the cells.

METHODS

Conditions of incubation in vitro. To maintain constant pH, to provide an adequate supply of glucose, to remove L(+)-lactate, to prevent infection and to maintain the normal plasma environment during incubations, whole blood was dialysed against a large volume of iso-osmotic nutritive buffer. Dialysis sacs were made from 36/32 in. Visking tubing that had been soaked in aq. 50% (v/v) ethanol for 48 hr., and in 1 mM-EDTA (sodium salt) for 48 hr. The sacs were rinsed and stored in ion-free water before use. A 2 ml. sample of heparin solution (2 mg. of sodium heparin B.P., containing no preservatives, per ml. of 0.9% NaCl) was placed in each sac. The sacs were then sterilized by autoclaving for 1 hr. in a domestic pressure cooker, in which they were kept until they were used.

A bicarbonate buffer was used, with additional ions added so as to simulate the inorganic components of blood plasma: Na⁺, 142.2 m-equiv./l.; Cl⁻, 119.7 m-equiv./l.; K⁺, 4.86 m-equiv./l.; HPO₄²⁻, 0.915 m-equiv./l.; Mg²⁺, 0.915 m-equiv./l.; HCO₃⁻, 27.4 m-equiv./l.; Ca²⁺, 0.915 m-equiv./l.; SO₄²⁻, 0.915 m-equiv./l.

D-Glucose (10g.) was added to 2180ml. of the buffer, which was made up in ion-free water. The buffer was placed in a 3l. conical flask in a water bath at 37°. A reflux condenser was used to prevent evaporation of the buffer. A gas mixture of CO₂+O₂ (5:95) was passed through the buffer to maintain pH 7.4. In most experiments the dialysis sacs were left stationary during incubation, and the red cells allowed to sediment. For each experiment about 15ml. of blood was taken from the antecubital vein, while occluding the venous circulation. Special care was taken in sterile precautions, and the sterility of the blood was frequently checked at the termination of the longer incubations. Blood was taken from a single donor throughout this study, except where otherwise indicated.

Osmotic fragility. Osmotic fragility of red cells was determined by the method of Dacie & Lewis (1963). The detailed results and their interpretation are described elsewhere (Gomperts, 1965). The osmotic fragility of red cells is commonly expressed as a statistical ogive (i.e. the integral of a frequency distribution). By plotting the osmotic fragility on arithmetic probability paper (Hunter, 1946), or by plotting probits on cartesian paper, it is possible to confirm the near normality of this distribution and to determine both the median red-cell fragility [concentration (w/v) of NaCl required to haemolyse 50% of the red-cell population] and the standard deviation of the red-cell population about the median. These two parameters have been used in this study to assess the tendency of red cells to haemolyse.

Red-cell Na⁺ and K⁺ ions. The intracellular concentrations of Na⁺ and K⁺ were determined by the method of Harris & Pranker (1953).

Methaemoglobin. Methaemoglobin, the ferric form of haemoglobin, was determined by the method of Evelyn & Malloy (1938).

Glucose consumption and lactate production. Fresh and incubated red cells, washed three times in iso-osmotic buffer (containing 1mg. of glucose/ml.), were placed in 50ml. round-bottom flasks fitted with Dreschel-type gas-washing tops. The buffer was added to the cells to bring them to a haematocrit of about 40%. The cells were continuously agitated by bubbling the CO₂+O₂ gas mixture (previously saturated with water by bubbling through cell-free buffer) through the suspension. The flasks were immersed in a water bath at 37°. After the cells had equilibrated with their environment (30min.), samples (3ml.) were withdrawn every 30min. from each flask. After centrifugation, glucose and lactate were measured in the supernatants.

Glucose was estimated by the Biochemica Boehringer test method 'Enzymatic determination of D-glucose with hexokinase/glucose-6-phosphate dehydrogenase'. The test mixture from the last sample (generally 3hr.) was used as a reagent blank, against which the earlier samples, containing more glucose and hence producing more NADPH, were compared.

Lactate was estimated by the Biochemica Boehringer test method 'Determination of the L-lactate concentration in blood or serum'. The test mixture from the first sample was used as a reagent blank, against which the later samples, containing more lactate and hence producing more NADH, were compared.

Phosphorylated glycolytic intermediates. The concentrations and the relative specific activities of intracellular

inorganic phosphate, ADP, ATP and 2,3-diphosphoglycerate were determined as follows. At the end of the incubation, the blood (about 15ml.) was placed in a 70ml. centrifuge tube. The cells were sedimented by centrifugation and samples of plasma were removed for determination of plasma lactate and inorganic phosphate. The cells were then resuspended and a sample was removed for the determination of haematocrit. The centrifuge tube was then placed in a 37° water bath and the blood gassed with the CO₂+O₂ mixture. After the blood had come to 37°, about 50μC of [³²P]phosphate (PBS 1; The Radiochemical Centre, Amersham, Bucks.) was added to the blood and mixed thoroughly. Exactly 0.1ml. of blood was diluted to 100ml. with water, to which a few drops of 1% ammonia solution had been added; this was preserved for radio-counting. After precisely 15min., the centrifuge tube was placed in an ice-water bath, and iced 0.9% NaCl added to the blood to fill the tube. After washing the cells four times with the cold 0.9% NaCl, the packed-cell volume was determined by using a micro-haematocrit centrifuge before preparing a protein-free extract with trichloroacetic acid (Bartlett, 1959a).

The specific activity of the plasma phosphate at the start of the exposure period was determined from the whole blood radiocount (counts/min.), haematocrit and plasma inorganic phosphate concentration.

Ion-exchange chromatography on Dowex 1 (X8; formate form) resin was used to separate the red-cell phosphorylated glycolytic intermediates. The buffer sequence of Morrell, Ayers & Greenwalt (1962) was used, the volumes being reduced for use with a 1cm.² × 12cm. column and smaller quantities of red cells (generally about 6ml.). This sequence was run as a series of three separate linear gradients of increasing concentration of formic acid and ammonium formate, interrupted after the second stage by a buffer of constant composition. For the first stage, water (40ml.) was placed in the mixing chamber and 0.1M-formic acid, pH 2.4 (40ml.), was placed in the reservoir. For the second stage 0.1M-formic acid (170ml.) was placed in the mixing chamber and a buffer containing 0.1M-formic acid plus 0.5M-ammonium formate, final pH 4.3 (170ml.), was placed in the reservoir. For the third stage a buffer containing 0.1M-formic acid plus 0.5M-ammonium formate, pH 4.3 (80ml.), was used. For the fourth stage a buffer containing 0.1M-formic acid plus 0.5M-ammonium formate, final pH 4.3 (100ml.), was placed in the mixing chamber, and a buffer containing 0.5M-formic acid plus 2.0M-ammonium formate, final pH 4.3 (100ml.), was placed in the reservoir. The buffers were pumped through the column at 28ml./hr. with a D.C.L. micro-pump, and fractions were collected every 15min. The column effluent was continuously monitored for radioactivity and extinction at 257mμ by using a programmed print-out counter (Labgear) and a mica end-window tube, in front of which the effluent was passed through a 50cm. Teflon tube, 0.6mm. internal diam., tightly wound on a small glass former, and a Locarte u.v. column scanner. A typical elution pattern showing radioactivity and u.v. absorption is shown in Fig. 1.

The following substances were identified: 1, AMP; 2, inorganic phosphate; 3, glucose 1,6-diphosphate; 4, fructose 1,6-diphosphate; 5, ADP; 6, 2,3-diphosphoglycerate; 7, ATP. The identities of these compounds were confirmed by using marker substances for column chromatography, and by the use of spectra, quantitative phosphate

Fig. 1. Chromatographic separation of red-cell acid-soluble phosphate compounds from Dowex 1 (X8; formate form) resin, by using formic acid and ammonium formate linear gradients. 1, AMP; 2, inorganic phosphate; 3, glucose 1,6-diphosphate; 4, fructose 1,6-diphosphate; 5, ADP; 6, 2,3-diphosphoglycerate; 7, ATP. —, ^{32}P radioactivity (counts/min.) detected by an end-window counter fitted with a flow cell; ----, $E_{257\text{ m}\mu}$ (arbitrary scale).

analysis and paper chromatography of the products of hydrolysis where appropriate. In addition, enzyme-catalysed reactions were used to confirm the identities of some of these substances. Red-cell ADP reacted with phosphoenolpyruvate and pyruvate kinase in the presence of NADH and lactate dehydrogenase. Red-cell fructose 1,6-diphosphate operated as a substrate in the reaction catalysed by aldolase, in the presence of NADH, triose phosphate isomerase and α -glycerophosphate dehydrogenase. Red-cell ATP reacted with 3-phosphoglycerate in the presence of phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase and NADH.

All the tubes comprising a peak were pooled and analysed to determine the mean specific activity and the total quantity of each compound per ml. of packed red cells.

Radioactivity counting. All radioactivity counts were performed by using Labgear electronics with a vertically mounted mica end-window counter, over a 1 ml. sample of liquid. This was placed in a drilled-out depression, 22.5 mm. diam. $\times$ 3 mm. deep, on a Perspex plate held at a fixed position under the mica end-window. At least 1000 counts were registered for each sample. The efficiency of counting

Fig. 2. Variation in the median of red-cell osmotic fragility during incubation *in vitro* of whole blood. The median of red-cell osmotic fragility is defined as the concentration of NaCl that induces the haemolysis of 50% of the red-cell population.

by this method was about 5%, the background about 12 counts/min.

Analysis of phosphorus. Esterified phosphorus was determined by the method of Fiske & Subbarow (1925) as modified by Bartlett (1959b). Inorganic phosphate was determined by the method of Michelson (1957).

Presentation of results. All quantities of intracellular phosphate compounds are expressed as $\mu\text{g. atoms of P/ml.}$ of packed cells and may be divided by 2 or 3 as appropriate for polyphosphorus compounds, to obtain concentrations in terms of $\mu\text{moles/ml.}$ of packed cells.

All radioactivity data are referred to the specific activity of the plasma inorganic phosphate at the start of the exposure period. Thus the mean relative specific activity of any compound is defined by: 100 (mean specific activity of intracellular compound)/(specific activity of plasma inorganic phosphate at the start of the exposure period).

RESULTS

Osmotic fragility. Fig. 2 shows the variation in median osmotic fragility of red cells during incubation *in vitro*. It can be seen that the sodium chloride concentration which induces 50% haemolysis increases very shortly after or at the moment of withdrawal of the blood. The main changes in this parameter are complete after about 30 hr. of incubation.

Fig. 3 shows the variation in log (standard deviation of osmotic fragility); this bears an approximately linear relationship with the period of incubation. The standard deviation increases only very slowly at first so that it is possible to use the variation in median osmotic fragility as a guide to overall changes in the cell population during the first few hours of incubation. The standard deviation increases more rapidly during the later hours of incubation, so that a comparison between the median fragility of fresh cells and cells

that have been incubated for 48 hr. is meaningless. Since the standard deviation of red cells continues to increase (at an ever-increasing rate) after the median osmotic fragility has stopped changing, it may be expected that a part of the cell population eventually becomes more resistant to osmotic haemolysis. This was observed after extended periods of incubation. Fresh cells are completely haemolysed (> 98%) by 0.3% sodium chloride, but the effect of this concentration of sodium chloride on cells that have been incubated for 72 hr. is to haemolyse only about 80% of the cell population.

Intracellular Na^+ and K^+ ions. The electrolyte concentrations of fresh cells were substantially constant during the first 12 hr. of incubation *in vitro*. The initial Na^+ concentration may be maintained for as long as 18 hr. (Fig. 4). Thereafter the Na^+ concentration increases, and the K^+ concentration decreases so that after about 84 hr. of

incubation the red-cell Na^+ concentration exceeds that of K^+ , although the condition of equilibration with the plasma is not yet approached.

The variation of red-cell Na^+ and K^+ concentrations contrasts with the variation in osmotic fragility, changes in the median of which are clearly detectable even after 3 hr.

Formation of methaemoglobin. The red-cell haemoglobin is very stable during incubation *in vitro*. No methaemoglobin was detected during the first 54 hr. Thereafter the concentration of methaemoglobin gradually increased so that after 96 hr. 7% of the total haemoglobin was oxidized to the ferric state. The presence of methaemoglobin has no deleterious effect on the spontaneous haemolysis of red cells (Jaffé, 1959; Vella, 1959).

Appearance of red cells after incubation. Fig. 5 shows a photomicrograph of a wet unstained preparation of blood after incubation for 96 hr. The cells are severely distorted and resemble acanthocytes: this is the only pathological condition in which the composition of the red-cell

Fig. 3. Variation in the standard deviation of osmotic fragility, expressed among the population of red cells, during incubation *in vitro* of whole blood.

Fig. 4. Variations in the concentrations of red-cell Na^+ and K^+ during the incubation *in vitro* of whole blood. O, Na^+ ; ●, K^+ .

Fig. 5. Appearance of red cells after incubation *in vitro* of whole blood for 96 hr. The red cells resemble acanthocytes; this is the only pathological condition in which the lipid composition of the red-cell membrane is known to be altered. Magnification: $\times 1100$.

Table 1. *Glucose consumption and lactate production by fresh red cells*

Fresh red cells were incubated at 37° in iso-osmotic bicarbonate buffer containing glucose (1 mg./ml.). Samples were removed every 30 min. and glucose and lactate were measured in the supernatant (see the Methods section).

Expt. no.	Glucose consumed (μ moles/hr./ml. of cells)	Lactate produced (μ moles/hr./ml. of cells)	Lactate/glucose ratio
1	1.29	2.44	1.9
2	1.33	2.51	1.89
3	1.29	2.67	2.07

membrane is known to be altered, with diminished lecithin and increased sphingomyelin content (Ways, Reed & Hanahan, 1961).

Rate of glucose consumption and lactate production.

(a) Fresh red cells suspended in the bicarbonate buffer consume about 1.3 μ moles of glucose/ml. of cells/hr., and produce about 2.5 μ moles of lactate/ml. of cells/hr. The consumption of glucose and the production of lactate under the experimental conditions is linear with time for 5 hr. The ratio of lactate production to glucose consumption is about 2:1, as would be expected for a system consuming glucose almost solely by anaerobic glycolysis (Table 1).

(b) After various periods of incubation, determinations of the rates of glucose consumption and lactate production indicate that there is an initial slight rise in the overall metabolism of the red cells after 24 hr. (see Fig. 6). Thereafter the rates of glucose consumption and lactate production decrease. The ratio of lactate production to glucose consumption decreases progressively from the start of incubation, settling at about 1.5:1 after 48 hr.

Studies on the phosphorylated metabolic intermediates of fresh red cells. (a) The quantities of inorganic phosphate, ADP, ATP and 2,3-diphosphoglycerate in fresh cells were determined on 16 separate occasions. The results are summarized in Table 2. The quantities found in the present study agree well with other data (e.g. Bartlett, 1959a).

(b) The mean relative specific activities of inorganic phosphate, ADP, ATP and 2,3-diphosphoglycerate after 15 min. exposure to [32 P]phosphate were determined on eight separate occasions. The results are summarized in Table 3. (The other eight experiments, included in Table 2, were conducted with 5 min. exposure periods, and are therefore not comparable with the rest of the work reported in this paper.) The scatter about the mean values is considerable and the results must be accepted with caution. It is worth noting that, whereas the variations in osmotic fragility, electrolytes and methaemoglobin were determined on small quantities of blood sampled after various periods of time from one incubation, the metabolic

Fig. 6. Variation in the rates of L(+)-lactate production and D(+)-glucose consumption by red cells after various periods of incubation *in vitro*. ●, L(+)-Lactate; ▲, D(+)-glucose.

experiments were performed on blood taken from one donor many times over 2 years. Drastic changes in the relative specific activities such as might accompany the eventual loss or accumulation of a compound during incubation of red cells would probably be apparent from these experiments.

Metabolic changes during incubation in vitro.

(a) Plasma. Though the pH and glucose concentrations were always perfectly equilibrated across the dialysis membrane, lactate accumulated in the plasma and never equilibrated perfectly. It could be shown, however, that the bulk of the lactate produced by the cells during incubation was dialysed into the buffer. The normal venous-plasma lactate concentration (under resting conditions, with no venous occlusion) is 1.28 ± 0.24 (s.d.) μ moles/ml. (Gibbs, Lennox, Nims & Gibbs, 1942). In the present study the lactate concentration of fresh blood was higher than this, because of venous occlusion and because the resting conditions were not applied. Table 4 shows that the concentration of plasma lactate rises sharply to a maximum after about 24 hr. of incubation, and it decreases slowly thereafter. In two experiments (72 hr. of incubation) in which the dialysis sac was kept in constant movement (the dialysis sac was moved up and down a distance of about 4 in., at

Table 2. Concentration of acid-soluble phosphate compounds in fresh red cells

A summary is given of 16 experiments performed on the blood of a single donor. Concentrations are expressed as $\mu\text{g. atoms of P/ml. of packed red cells}$.

	P_i	ADP	ATP	2,3-Diphospho- glycerate
Mean concentration	0.47	0.50	3.36	8.97
Standard deviation	0.009	0.009	0.32	0.5
Coefficient of variation	1.8%	1.8%	9.5%	5.8%

Table 3. Mean relative specific activities of acid-soluble phosphate compounds of fresh red cells after exposure of whole blood to $[^{32}\text{P}]$ phosphate for 15 min.

The specific activities are expressed as a percentage of the specific activity of the plasma inorganic phosphate at the commencement of the exposure period. A summary is given of eight experiments.

	P_i	ADP	ATP	2,3-Diphospho- glycerate
Mean relative sp. activity	3.77	1.76	2.2	0.26
Standard deviation	0.83	0.33	0.4	0.0056
Coefficient of variation	22%	19%	18%	2.2%

Table 4. Variation of plasma lactate during incubation in vitro of red cells

Time of incubation (hr.)	Concn. of plasma lactate ($\mu\text{moles/ml.}$)
0	1.74
24	7.21
42	6.45
48	5.67
66	2.95
72	3.32
96	2.06

about 1 cycle/min.) the plasma lactate concentration was maintained at a much lower level.

The plasma inorganic phosphate concentration also tended to increase during the early hours of incubation, possibly declining after very long periods. In several instances, however, the plasma phosphate concentration at intermediate times was the same as for fresh blood; this may be due to variation in the efficacy of dialysis.

(b) Red cells. The results reported in this section are devoted to trends over extended periods of incubation.

Inorganic phosphate. During the first 18 hr. of incubation *in vitro* the intracellular inorganic phosphate concentration rises from the initial mean value of $0.47 \mu\text{g. atom of P/ml. of cells}$ to about $1.5 \mu\text{g. atoms of P/ml. of cells}$. This is illustrated in Fig. 7(a). After 18 hr., the scatter of experimentally determined quantities was greater, but it seems probable that the high concentration achieved after 18 hr. is generally maintained thereafter.

Fig. 7. Variation in the concentrations of red-cell inorganic phosphate (a) and ATP (b) during incubation *in vitro* of whole blood.

ADP and ATP. During incubation, the red-cell ATP concentration falls from the initial mean value of $3.4 \mu\text{g. atoms of P/ml. of cells}$ so that after 96 hr. about half the initial quantity remains. The rate of loss of ATP is faster at the start of incubation than later, but the loss is continuous even in the later stages of incubation (see Fig. 7b). The concentration of red-cell ADP probably declines also during incubation *in vitro*. The mean initial concentration of ADP is $0.5 \mu\text{g. atom of P/ml. of cells}$; after 96 hr. of incubation the mean concentration of ADP was $0.22 \mu\text{g. atom of P/ml. of cells}$ (three experiments).

Fig. 8. Loss of 2,3-diphosphoglycerate from red cells during incubation *in vitro* of whole blood in a dialysis system. In two experiments (Δ , circled) the blood was shaken during a 72 hr. incubation period, and the loss of 2,3-diphosphoglycerate was retarded.

Fig. 9. Variation in the relative specific activity of red-cell 2,3-diphosphoglycerate during incubation *in vitro* followed by exposure to $[^{32}\text{P}]$ phosphate for 15 min. at 37° .

2,3-Diphosphoglycerate. During incubation *in vitro* of whole blood, the concentration of red-cell 2,3-diphosphoglycerate decreases rapidly (Fig. 8). Fresh red cells have a 2,3-diphosphoglycerate concentration of $9 \mu\text{g. atoms of P/ml. of cells}$. After 15 hr., only about half this quantity remains. A steady concentration of about $0.4 \mu\text{g. atom of P/ml. of cells}$ is achieved after about 48 hr. and this concentration does not thereafter decline. In two experiments in which the dialysis sac was kept in motion during the incubation period to improve the equilibration across the dialysis membrane, the concentration of 2,3-diphosphoglycerate after 72 hr. was 4.9 and $4.7 \mu\text{g. atoms of P/ml. of cells}$. This is more than ten times the concentration otherwise found after this time and about the concentration expected after 12 hr. of incubation.

As shown in Fig. 9, the mean relative specific activity of red-cell 2,3-diphosphoglycerate increases from an initial value of about 0.26% to about 0.5% during the first 15 hr. of incubation. The relative

specific activity may continue to rise slightly above 2% during the later hours of incubation, but the main changes are probably complete after 48 hr., the time after which an essentially steady concentration of 2,3-diphosphoglycerate is maintained.

DISCUSSION

The first changes detected in red cells after withdrawal of whole blood and incubation *in vitro* are an increase in the median of osmotic fragility and a loss of 2,3-diphosphoglycerate. After longer periods of incubation the cells suffer a gradual decrease in their overall metabolic rate and a decrease in the ratio of the rates of lactate production and glucose consumption. There is a decrease in the intracellular concentration of adenine nucleotide, and an exponential increase in the standard deviation of osmotic fragility expressed among the population of red cells.

A loss of 2,3-diphosphoglycerate from red cells incubated as whole heparinized blood in silicone-treated glass vessels in the presence of glucose was reported by Pranker & Altman (1954). The loss of 2,3-diphosphoglycerate also occurs when washed red cells are suspended in phosphate-buffered saline in the presence of glucose in glass vessels (Whittam, 1958). In the latter study it was shown that 2,3-diphosphoglycerate is quantitatively converted into pyruvate. Most of the red-cell 2,3-diphosphoglycerate is probably formed directly from 1,3-diphosphoglycerate in the reaction catalysed by diphosphoglycerate mutase (1,3-diphospho-D-glyceric acid-3-phospho-D-glycerate phosphotransferase, EC 2.7.5.4) (Rapoport & Luebering, 1950), and is probably destroyed in the reaction catalysed by diphosphoglycerate phosphatase (2,3-diphospho-D-glycerate 2-phosphohydrolase, EC 3.1.3.13) (Rapoport & Luebering, 1951) in which the products are inorganic phosphate and 3-phosphoglycerate. A high steady-state concentration of 2,3-diphosphoglycerate is maintained *in vivo* (about 60% of red-cell phosphorus); on withdrawing and incubating, the steady-state condition is soon upset. The resulting loss of red-cell 2,3-diphosphoglycerate may arise from a slowing up in its rate of synthesis, or from an accelerated rate of destruction. The present experiments were designed to distinguish between these two alternatives.

The pattern of increasing relative specific activity with concurrent loss of 2,3-diphosphoglycerate in an approximately inverse relationship indicates that the loss of this compound is due to an accelerated rate of destruction, while its rate of synthesis remains approximately constant. The loss of 2,3-diphosphoglycerate from red cells when whole blood is incubated *in vitro* may be due to

activation of the reaction catalysed by diphosphoglycerate phosphatase; it is necessary to consider why this should occur. The retardation of the loss of 2,3-diphosphoglycerate when the blood was incubated with shaking indicated that efficient dialysis of the blood prevents the activation of this process. Since it was shown that lactate accumulated in the blood during stationary incubation, but is removed when the dialysis sac is shaken, it is possible that lactate or some other small molecule present in venous blood is responsible for the activation of diphosphoglycerate phosphatase. Further, the observation that 2,3-diphosphoglycerate disappears from washed red cells suspended in plasma-free buffer (Whittam, 1958) indicates that the molecule activating this reaction is produced by the red cells themselves. These possibilities are being investigated.

The increase in the median of red-cell osmotic fragility follows a similar time-course to the loss of 2,3-diphosphoglycerate from the cells. It is noteworthy that the loss of a non-diffusible substance is accompanied by a greater tendency for the critical haemolytic volume of the cells to be exceeded, over a period during which no other changes of comparable magnitude were observed.

The increase in the concentration of intracellular inorganic phosphate is probably due to the breakdown of 2,3-diphosphoglycerate. However, the increase of inorganic phosphate represents only a small part of the 2,3-diphosphoglycerate destroyed, and the main part must be lost into the plasma and thence into the buffer. The low concentration of plasma inorganic phosphate has been shown to limit the rate of the phosphofructokinase-catalysed reaction in red cells (Minakami, Kakinuma & Yoshikawa, 1964); 2,3-diphosphoglycerate has been shown to inhibit red-cell hexokinase (Dische, 1940). It is possible that the simultaneous loss of 2,3-diphosphoglycerate and gain of inorganic phosphate causes the slight increase in the rate of glycolysis after 24 hr.

The reasons for the loss of adenine nucleotide from incubated red cells were not clarified by these experiments as the scatter of relative specific activities of ATP and of ADP was too wide to allow a clear-cut distinction between decreased synthesis and accelerated destruction. However, red cells are not known to synthesize adenine compounds, and a loss of total adenine nucleotide was observed. Therefore it seems likely that the disappearance of adenine nucleotide is due to a process of accelerated destruction. It was shown that beyond 24 hr. of incubation there is a decreased rate of glucose consumption and therefore a decreased rate of phosphorylation of ADP is reasonable. An active 5'-adenylate deaminase has been found in blood (Conway & Cooke, 1939) and

it is possible that red cells lose adenine nucleotide by deamination to inosinic acid, although no systematic changes in the nucleoside 5'-monophosphates were found. There is evidence that the deterioration of blood stored in acid-citrate-glucose medium may be due to activation of one of the enzymes catalysing the sequence of reactions from ATP to hypoxanthine (Bishop, Rankine & Talbott, 1960), and the observation that 5'-adenylate deaminase from muscle is activated by lactate (as well as chloride, acetate and citrate) (Nikiforuk & Colowick, 1955) makes it appear likely that the loss of adenine nucleotide in incubated red cells is due to activation of 5'-adenylate deaminase due to the increased concentration of lactate, which accumulated in the plasma during incubation. Dialysis of hypoxanthine may assist this process.

The concentration for half-maximal activation by ATP of membrane Na^+ -plus- K^+ -dependent adenosine triphosphatase is 0.5 mM (Post, Merritt, Kinsolving & Albright, 1960); the ATP concentration of fresh red cells is twice this, and it might therefore be expected that the activity of this enzyme would be sensitive to alteration in the concentration of ATP, and that this might be reflected in the cation distribution ratios. The retention of the initial Na^+ and K^+ concentrations during the first 18 hr. of incubation (during which time the ATP concentration falls from 3.5 to 2.5 $\mu\text{g. atoms of P/ml. of cells}$) indicates that the sequence of cause and effect is not so straightforward. It is of course the rates of Na^+ and K^+ transport that are affected directly by the adenosine-triphosphatase reaction (Whittam, 1958), but these have not been investigated in the present study. The loss of K^+ from red cells during longer incubations was never fully compensated by an equivalent gain in intracellular Na^+ . This imbalance may, however, reflect the loss of intracellular anion (adenine nucleotides and 2,3-diphosphoglycerate) that occurs during incubation.

The systematic variation in the standard deviation of osmotic fragility is intriguing and raises other basic questions about the distribution of characteristics among the cell population; e.g. does the standard deviation of the rate of cation transport about its mean increase exponentially with time of incubation as well? This and other questions remain unanswered, and for the present the exponential increase in the standard deviation of osmotic fragility with time stands out as an isolated observation. One may suggest that it is due to a biphasic response by different sections of the red-cell population to a single chemical change within the cells. In the absence of further experimental data, this and other plausible explanations remain speculative.

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